Vol. 89: 9–15, 2010
doi: 10.3354/dao02182

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published February 24

Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control

Peter-Joachim Enzmann[1,*], Jeannette Castric[2], Giuseppe Bovo[3], Richard Thiery[2,6], Dieter Fichtner[4], Heike Schütze[4], Thomas Wahli[5]

[1]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Paul-Ehrlich-Str. 28, 72076 Tübingen, Germany
[2]Afssa-site de Ploufragan/Brest, Technopôle Brest-Iroise, BP 70, 29280 Plouzané, France
[3]Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD), Italy
[4]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Suedufer 10, 17493 Greifswald - Insel Riems, Germany
[5]National Fish Disease Laboratory, Centre for Fish and Wildlife Health, Institute of Animal Pathology, University of Bern, POB 8466, 3001 Bern, Switzerland
[6]*Present address*: Afssa-site de Sophia-Antipolis, BP 111, 06902 Sophia-Antipolis, France

ABSTRACT: The fish pathogenic rhabdovirus infectious hematopoietic necrosis virus (IHNV) causes substantial losses in European aquaculture. IHNV was first detected in Europe in 1987 and has since undergone considerable spread. Phylogenetic analyses of the full G-gene sequences of 73 isolates obtained from 4 countries in Europe (France, n = 18; Italy, 9; Switzerland, 4; Germany, 42) enable determination of the evolution of the virus in Europe since the first detection, and identification of characteristic changes within the G-genes of European strains. Further, the database allows us to analyse the pathways of distribution in Europe over time. The results suggest that in most of the recent cases, spread of IHNV was related to trade of infected fish. The data further demonstrate that knowledge of the sequence is required to determine the source of infections in farms.

KEY WORDS: IHNV evolution · Phylogenetic tree · Control

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV), the causative agent of infectious hematopoietic necrosis, belongs to the rhabdovirus genus *Novirhabdovirus* (Morzunov et al. 1995, Pringle 1999, Enzmann 2000, Fauquet et al. 2005). IHN was originally observed as a disease mainly of Pacific salmon and trout in enzootic areas in western North America. In 1987, IHNV was detected for the first time in Europe, in France and Italy (Baudin-Laurencin 1987, Bovo et al. 1987). In 1992, IHNV was isolated from rainbow trout *Oncorhynchus mykiss* in Germany, the same host species as in France and Italy (Enzmann et al. 1992). Since then, IHN has become a serious threat to the European rainbow trout farming industry, especially in various parts of France, Italy and Germany. The fish-farming industry in Europe is mostly private; there are only a few governmental hatcheries for the purpose of stocking wild waters. The capacity of the private fish farms varies from a few tons (t) yr^{-1} to 100 t or more.

Prevention and control of infectious diseases in fish farms, as well as movements of live aquaculture animals between member states, are regulated in the European Union (EU; Council of the European Union 2006). IHN is a compulsorily notifiable disease. Dependent on the health status of aquaculture zones or compartments, 5 categories are defined: I, disease-free; II, surveillance programme; III, undetermined; IV, eradication programme; and V, infected. Fish may only be

*Email: peter-joachim.enzmann@fli.bund.de

introduced into Category I farms from farms of Category I. The same is valid for Category II and IV farms. Category III farms may introduce fish from Category I, II and III farms. Category V farms may introduce fish from all categories. The status 'disease-free' is controlled every year by the competent authority. In the case of suspicion of IHN, samples are taken and examined in an authorised laboratory, the farms are placed under official surveillance and control measures are implemented to prevent spread of the disease. An epizootic investigation to detect the source of the infection and to determine whether fish have left the farm preceding the notification of the suspicion is initiated. In the case of confirmation of IHN, the farm is officially declared infected; a containment area is established, no restocking takes place and no fish are moved into, within or out of the containment area. Switzerland enacted laws corresponding to those of the EU-member states France, Italy and Germany. In particular, confirmation of infected fish in a farm results in immediate stamping out of the stocks.

For IHNV, 3 major virus genogroups designated U, M and L, indicating their general correlation with the upper, middle and lower portions, respectively, of the IHNV geographical distribution in North America (Kurath et al. 2003) have been determined. Analyses of the European isolates revealed an affiliation to Genogroup M, and Enzmann et al. (2005) suggested that all isolates were progenies of a virus introduced into France and Italy in 1987. In the present study we report on the further distribution and development of IHNV in Europe by comparing the complete nucleotide sequences of the G genes from European isolates originating from France (18 isolates), Italy (9 isolates), Switzerland (4 isolates), and Germany (42 isolates).

MATERIALS AND METHODS

Virus isolates. Virus strains were obtained from national reference laboratories by the authors (France: J.C.; Italy: G.B.; Switzerland: T.W.; Germany: D.F.) or from German regional fish disease laboratories (Table 1). All IHNV isolates were obtained as virus in frozen cell-culture supernatants prepared in accordance with standardized fish health protocols from the EU (Commission of the European Communities 2001).

The origin and sequences of IHNV reference strains RB, WRAC and SRCV, as well as isolates D332-92, Dfs62-95, Dfs42-95, Dfs30-95, Dfs13-98, Dfs8-99 and Dfr100-96 were described earlier (Enzmann et al. 2005). The new virus isolates used in the present study were not cloned in order to preserve natural quasispecies diversity. All analysed viral sequences, 73 in total, are summarized in Table 1. All virus isolates originated from fish farms rearing rainbow trout, except one (Dfs13-98), which was isolated from wild eel. Virus isolates were obtained after outbreaks of IHN or during routine inspections, in which cases samples from fish were taken.

RT-PCR and sequence analysis. RNA isolation and RT-PCR were performed as described previously (Enzmann et al. 2005). The primers IM2WS (5′-ACT ACT ATG CCC AGG AGA CA-3′) and ILZA (5′-TTC CGC TGG AAG TCT CTC TT-3′) were used for the amplification of a fragment from the end of the matrix protein gene M to the start of the viral RNA polymerase gene L. The resulting RT-PCR products were 2299 nucleotides (nt) in length and comprised the region from nt 2780 to nt 5078, of the complete IHNV genome (GenBank accession no. X89213, Schütze et al. 1995). All sequences were confirmed by sequencing the PCR products in both orientations.

The phylogenetic studies were performed with the nucleotide sequence of the complete open reading frame (ORF) encoding for the IHNV glycoprotein G (nt 3007 to 4533; X89213, Schütze et al. 1995). For sequence alignments and phylogenetic analyses, the PAUP program (gcg, Wisconsin Package) was applied using bootstrap analysis with heuristic tree search and maximum parsimony. Bootstrap values exceeding 70% were considered to indicate significant relatedness. The complete nucleotide sequences of IHNV glycoprotein genes from European isolates presented here were submitted to the National Center for Biotechnology Information (NCBI) database. The respective GenBank accession numbers are listed in Table 1.

RESULTS

IHNV isolates and phylogenetic tree

To investigate the development of IHNV in Europe, we determined the nucleotide sequence of the G gene ORF from 73 European isolates originated from France, Italy, Switzerland and Germany between 1987 and 2007. Table 1 shows the list of viral isolates used in this study. These viruses were gained from outbreaks of IHN, or from routine examinations prescribed by EU legislation. All isolates represent different collection sites (one exception, see Subclade F). The evolution of European IHNV over the 20 yr period since the first isolation in Italy and France in 1987 is illustrated in the phylogenetic tree shown in Fig. 1; the results of this study are summarised in Table 2. For comparative reasons, the North American reference strains WRAC, RB and SRCV, belonging to Genogroups M, U and L, respectively, were included in this study. The nomen-

Table 1. Infectious hematopoietic necrosis virus (IHNV). Virus isolates used in this study. 1FO: first French outbreak; 1IO: first Italian outbreak; G ORF: G gene open reading frame. Prefixes: F, French isolates; CH, Swiss isolates; D, German isolates; I, Italian isolates. RB, WRAC and SRCV are reference isolates from North America

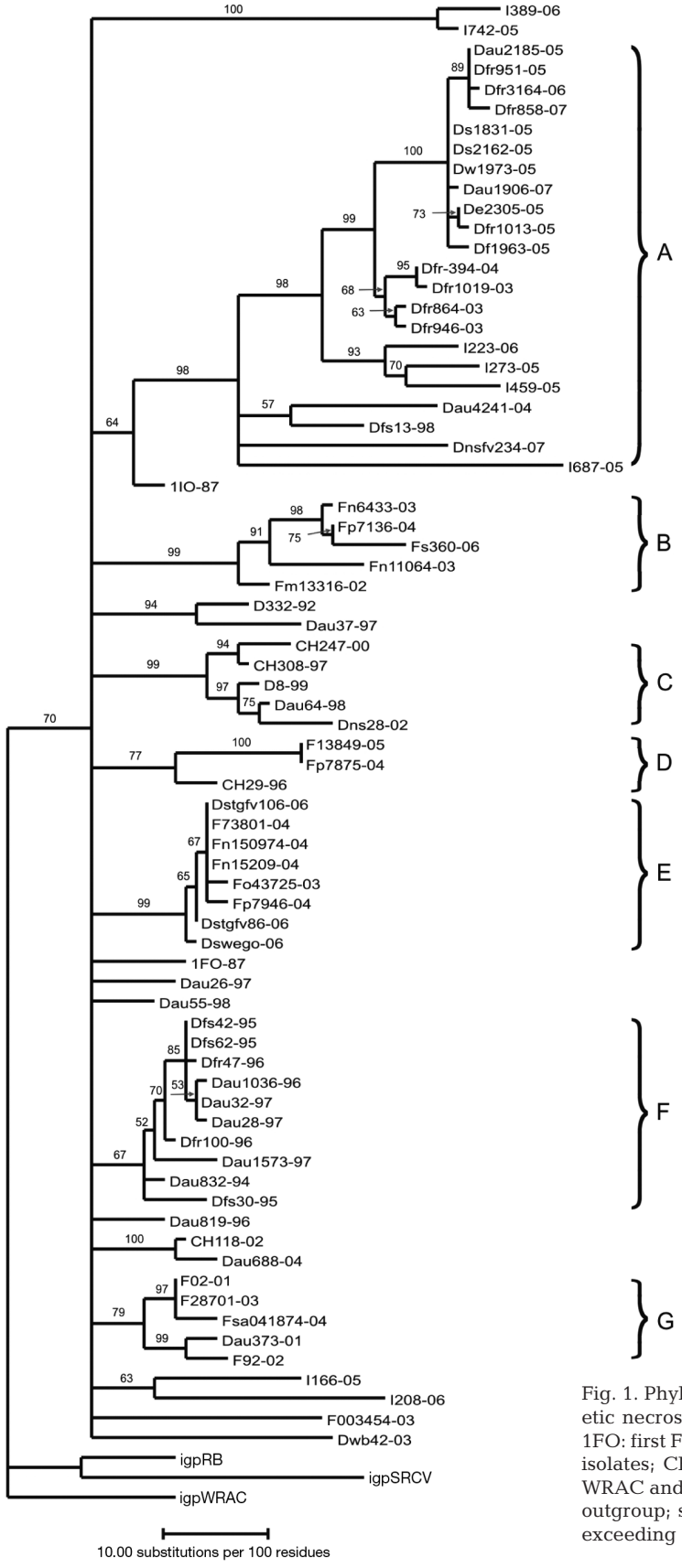
Virus isolate	G ORF accession	Year of isolation
1FO-87	X89213	1987
1IO-87	FJ711518	1987
F02-01	EU331442	2001
F92-02	EU331443	2002
F003454-03	EU331444	2003
F73801-04	EU331445	2004
F28701-03	EU331446	2003
Fm13316-02	EU331447	2002
Fn6433-03	EU331448	2003
Fn11064-03	EU331449	2003
Fn15209-04	EU331450	2004
Fn150974-04	EU331451	2004
Fo43725-03	EU331452	2003
Fp7136-04	EU331453	2004
Fp7875-04	EU331454	2004
Fp7946-04	EU331455	2004
Fsa041874-04	EU331456	2004
F13849-05	EU676229	2006
Fs360-06	EU676237	2006
CH29-96	EU676196	1996
CH118-02	EU676197	2002
CH247-00	EU676198	2000
CH308-97	EU676199	1997
Dau26-97	EU676200	1997
Dau28-97	EU676201	1997
Dau32-97	EU676202	1997
Dau37-97	EU676203	1997
Dau55-98	EU676204	1998
Dau64-98	EU676205	1998
Dau373-01	EU676206	2001
Dau688-04	EU676207	2004
Dau819-96	EU676208	1996
Dau832-94	EU676209	1994
Dau1036-96	EU676210	1996
Dau1573-97	EU676211	1997
Dau1906-07	EU676212	2007
Dau2185-05	EU676213	2005[a]
Dau4241-04	EU676214	2004
De2305-05	EU676215	2005[a,b]
Df1963-05	EU676216	2005[a,b]
Dfr100-96	EU676217	1996
Dfr858-07	EU676218	2007[a]
Dfr864-03	EU676219	2003[a]
Dfr946-03	EU676220	2003
Dfr951-05	EU676221	2005
Dfr1013-05	EU676222	2005[a]
Dfr1019-03	EU676223	2003
Dstgfv86-06	EU676224	2006[a,b]
Dstgfv106-06	EU676225	2006
Dswego-06	EU676226	2006[a]
Dw1973-05	EU676227	2005
Dwb42-03	EU676228	2003
Dfr3164-06	EU676230	2006[a]
Dfr394-04	EU676231	2004[b]
Dfr47-96	EU676232	1996
Dns28-02	EU676233	2002
Dnsfv234-07	EU676234	2007
Ds1831-05	EU676235	2005
Ds2162-05	EU676236	2005
I166-05	FJ711510	2005
I208-06	FJ711511	2006
I223-06	FJ711512	2006
I273-05	FJ711513	2005
I389-06	FJ711514	2006
I459-05	FJ711515	2005
I687-05	FJ711516	2005
I742-05	FJ711517	2005
D332-92	AY331657	1992
Dfs13-98	AY331658	1998
Dfs8-99	AY331660	1999
Dfs30-95	AY331662	1995
Dfs42-95	AY331663	1995
Dfs62-95	AY331664	1995
RB	Reference isolate	1976
WRAC	Reference isolate	1982
SRCV	Reference isolate	1966

[a]Virus transmission by infected fish
[b]Heterogeneity detected

clature of isolates is composed of an indicator for the European country of origin (F: France; I: Italy; CH: Switzerland; D: Germany), the specific identification number of the respective laboratory and the year of isolation following a hyphen.

Although not strongly supported (bootstrap value 67), Subclade F (Fig. 1) with the isolates from Dfs42-95 to Dfs30-95 is interesting because it represents only German strains isolated in the period from 1994 to 1997. Strains Dfs42-95 and Dfs62-95 are identical, but were obtained from 2 different farms in the same region (about 20 km apart). Strain Dfr47-96 persisted in one of these farms and was isolated in the following year; it differed from the original virus in 1 nt. Isolates with this genetic affiliation were never again detected in Germany after the application of radical measures in 1997. In these fish farms, farmers were not allowed to remove live fish after the first suspicion of infection, and stamping out and disinfection were effected after diagnosis; these measures were accompanied by controlling those farms to which fish were delivered from the diseased farm prior to the suspicion of infection, and effecting the same measures if virus transmission was verified.

Other groups clustered within the tree represent IHNV isolates from different European origins. The main sector from Dau2185-05 to I687-05 (Subclade A, Fig. 1) includes German and Italian isolates found from 1998 to 2007. The first Italian isolate, 1IO-87, is basal to Subclade A, although with weak bootstrap support.



The year of isolation indicates that these strains are involved in the current circulation of IHNV in Germany. A more detailed analysis of transmission of IHNV between farms using the viruses circulating within a limited region in Germany is discussed later (Fig. 2). Adjacent to this group is a small subclade containing only French isolates detected from 2002 to 2006 (Fn6433-03 to Fm13316-02, Subclade B). The group from CH247-00 to Dns28-02 (Subclade C), observed between 1997 and 2002, demonstrates the distribution of a group of related viruses within Germany and Switzerland. The relation of Swiss IHNV to French isolates was demonstrated in the following cluster (Subclade D), in which the closely related French isolates F13849-05 and Fp7875-04 are grouped together with an older IHNV isolate detected in Switzerland in 1996.

In addition to the current situation of common viruses within Italy and Germany, France and Switzerland, and Germany and Switzerland, the following cluster (Subclade E, Fig. 1) contains a group of IHNV isolates circulating from 2003 until 2006 in France and Germany (Dstgfv106-06 to Dswego-06). Similarly, another subgroup (Subclade G), containing 5 viruses isolated from France and Germany in the years 2001 to 2004 with a bootstrap value of 79 is defined. From the summary of these results in Table 2, it is visible that Subclade A contains viruses actually circulating in Germany, whereas Subclade F viruses disappeared; further, a remarkable trend in crossing country boundaries is notable.

Pathway of distribution of an IHNV-subgroup

A detailed phylogenetic analysis of the events during circulation of a specific virus type within several independent private farms in the southern part of Germany is given in Fig. 2. This tree is part of Subclade A in Fig. 1. In Subclade A, a further subgroup (bootstrap value 100) comprises the German isolates Dau2185-05 to Dfr1963-05, which played a major role in an epizootic within a limited region in Germany. The geographic distribution of the viruses is shown in Fig. 3. Within this group, the

Fig. 1. Phylogenetic tree showing the evolution of infectious hematopoietic necrosis virus (IHNV) within about 20 yr of circulation in Europe. 1FO: first French outbreak; 1IO: first Italian outbreak; Prefixes: F, French isolates; CH, Swiss isolates; D, German isolates; I, Italian isolates. RB, WRAC and SRCV are reference isolates from North America used as the outgroup; sequences were not delivered to GenBank. Bootstrap values exceeding 70% were considered to indicate significant relatedness. A–G: subclades

Table 2. Infectious hematopoietic necrosis virus (IHNV). Summary of the phylogenetic study. F: France; CH: Switzerland; D: Germany; I: Italy

Subclade	Bootstrap (%)	No. of isolates	Country	Years of isolation
A	98	22	D, I	1998, 2003–2007
B	99	5	F	2002, 2003, 2006
C	99	5	D, CH	1997–2000, 2002
D	77	2	F	2004, 2005
E	99	8	D, F	2003, 2004, 2006
F[a]	67[b]	10	D	1995–1997
G	79	5	F, D	2001–2004
Not clustered		20	D, F, I, CH	1987, 1992, 1996–1998, 2001–2006

[a]Virus type no longer detected after stamping out
[b]Bootstrap value below 70

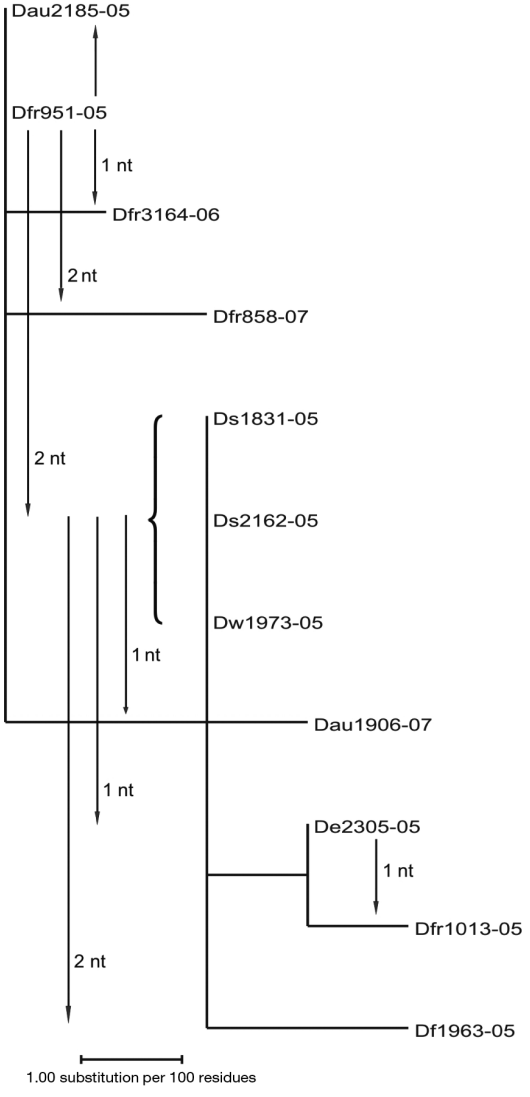


Fig. 2. Phylogenetic tree demonstrating the circulation of specific infectious hematopoietic necrosis virus (IHNV) strains within the southern part of Germany. The relationship of the virus isolates is shown by arrows and the numbers of differing nucleotides (nt)

infection cycle could be partially identified and transfer of virus documented starting from isolate Dfr951-05. This virus was transmitted from the first farm to 3 other farms. From these 3 farms, the following viruses could be isolated: Dau2185-05, which was identical to Dfr951-05 in the same year, 2005, and Dfr3164-06 isolated in 2006, which showed a single mutation, and Dfr858-07 in 2007, which revealed 2 mutations (Figs. 2 & 3). In the same year in which Dfr951-05 was isolated, the virus was also transmitted to 3 additional farms in which viruses Ds1831-05, Ds2162-05 and Dw1973-05 were found. Fish trade records of these transfers could not be documented unequivocally (dashed lines in Fig. 3). The latter 3 farms are in the same region, located about 10 km apart from each other. The 3 viruses isolated from these 3 farms are identical but differ from the original virus (Dfr951-05) by 2 mutations (nt 199 and nt 1513). Virus Dw1973-05 was transmitted to 2 additional farms resulting in viruses De2305-05 (another farm belonging to the same owner) and Df1963-05, differing in 1 and 2 nt, respectively. Virus De2305-05 was further transmitted to a farm in which virus Dfr1013-05 was isolated, differing in 1 nt (Fig. 3).

DISCUSSION

In contrast to the previously used principle to clone all virus strains by at least 4 end-point titrations before sequencing (Enzmann et al. 2005), the new virus isolates sequenced for the present study were not cloned in order to detect quasi-species variation. The computed sequences were checked manually. In several consensus sequences a particular nt-peak was revealed to be a double peak, i.e. a smaller peak was located underneath the main peak (with corresponding occurrence in the complementary DNA strand). For these cases, the following interpretation was adopted: when the consensus sequence differed from a known sequence exactly in this particular nt, the consensus sequence was defined as a new virus strain descended from the known strain. The sequence of the original strain is hidden in the computed consensus sequence, because the field isolate is a mixture of the known virus strain and a mutated strain with the mutated strain in the majority. Thus, the ancestry of a new virus strain is determined (marked in Table 1 by superscript 'b'). Using this method, current trends in the development of new virus strains can be discov-

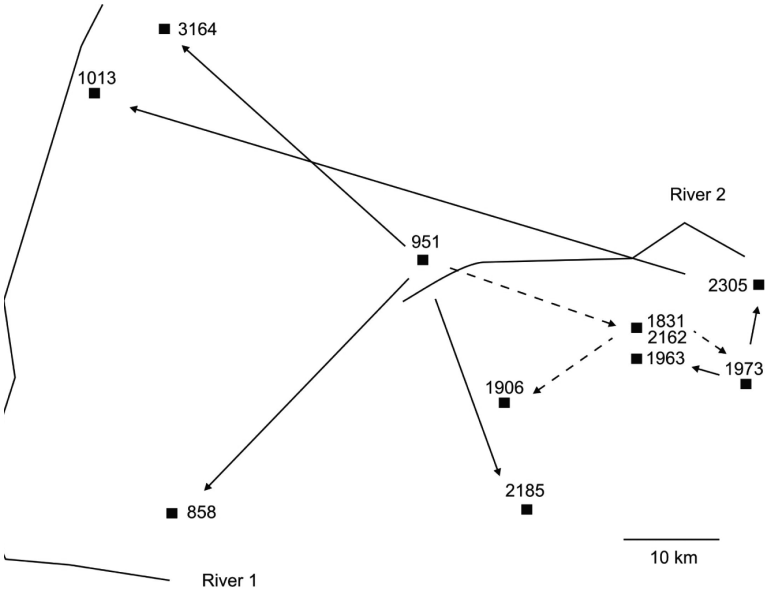


Fig. 3. Infectious hematopoietic necrosis virus (IHNV). Outline of the geographical relationship of outbreaks analysed in Fig. 2. Numbers represent virus isolates. Lines without arrows indicate rivers. Arrows indicate direction of transfer. Dashed lines mean that fish transport records were not documented unequivocally

ered at an earlier stage than with previously used methods. This also reveals that if field virus isolates are cloned after isolation, information on the development of the virus may be lost.

After 2003, intensive studies on the epidemiology and phylogeny of IHNV were initiated in Germany. The results of these analyses revealed that in the majority of IHN outbreaks the transmission of virus had occurred by trade of infected fish. This can also be concluded from the clustering pattern of German strains isolated after 2003 and is further supported by the fact that new outbreaks occurred after partial restocking of farms. It was possible in almost all cases to identify the origin of IHNV and the means by which it was introduced into a fish farm, and factors other than trade could be involved, e.g. transmission by birds. It was clearly demonstrated that IHNV travels through Europe apparently without significant restrictions. Closely related viruses found in Germany and France, in Germany and Switzerland, in France and Switzerland, as well as in Italy give evidence for this viral 'tourism' (Fig. 1, Table 2). The precise method of crossing borders remains to be determined by fish health officials. European regulations on the control of fish and fish egg transportation are clearly defined (Council of the European Union 2006), but nevertheless, the vast majority of fish farming in central Europe is private and violations cannot be excluded. The reason for frequent fish transport between private farms is governed by the law of supply and demand. Even small differences between prices can give rise to fish trade between private farms.

Phylogenetic analysis is revealed to be the most effective method of elucidating the ways of dissemination and evolution of IHNV and the correlation of virus distribution with trade channels. However, it could also be clearly demonstrated (Subclade F, Fig. 1) that strict sanitary measures eliminate IHNV, since after the application of rigorous measures in1997, isolates with this specific genetic make-up were never again detected in Germany.

From this study, it can be further concluded that development of IHNV may be very rapid in some cases. Within 1 year (2005), up to 4 mutations within the glycoprotein gene of IHNV occurred during the infection cycle in a limited region (Dfr951-05 and Df1963-05, Fig. 2), whereas in other cases, 1 (Dfr3164-06), 2 (Dfr858-07) and 3 (Dau1906-07) mutations occurred within 1 and 2 years.

The main risk of IHN outbreaks is the introduction of infected fish into a fish farm. Based on the guidelines of the World Organisation for Animal Health (OIE) and the EU (Council of the European Union 2006), IHN is a notifiable disease. In the EU, control measures are based on the isolation of the causal agent followed by serological identification. However, so far only sequence analyses of regions of the viral genome offer the capability of identifying and differentiating the isolates, thus providing a powerful tool in epidemiological investigations. Therefore, it is recommended that all newly detected IHNV isolates should be sequenced. Direct sequencing analyses of RT-PCR products provide the necessary information to identify new trends in the development of virus strains.

Acknowledgements. We thank A. Braun for excellent technical assistance.

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Editorial responsibility: Mark Crane, Geelong, Victoria, Australia

Submitted: July 2, 2009; Accepted: November 12, 2009
Proofs received from author(s): February 8, 2010